PRO-OPIOCORTIN CONVERTING ACTIVITY IN RAT INTERMEDIATE AND NEURAL LOBE SECRETORY GRANULES

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1. Introduction

The intermediate lobe of the pituitary of many species synthesizes adrenocorticotropin (ACTH), β-lipotropin (β-LPH), α-melanotropin (α-MSH) and β-endorphin (β-END). Biosynthesis studies have shown that these peptides are cleaved from a glycosylated common prohormone, pro-opiocortin (M_r 32 000, see fig.1) [1–4]. The primary structure of bovine pro-opiocortin has been derived from the nucleotide sequence of the cloned cDNA of the molecule [5], and the amino acid sequence revealed pairs of basic amino acids separating each of the peptides to be cleaved (see fig.1) similar to other prohormones [6]. Therefore, it has been proposed that processing of pro-opiocortin requires a trypsin-like enzyme to cleave at the basic amino residues, followed by the action of a carboxypeptidase B-like enzyme to remove the basic amino acid from the C-terminus. Studies indicate that the processing occurs intragranularly [7,8] and therefore such enzymes are likely to be localized within the secretory granules.

We had showed the presence of a pro-opiocortin converting activity, that cleaves specifically at pairs of basic amino acids, in rat pituitary neurointermediate lobe secretory granules [9]. In [9], characterization of the activity using different protease inhibitors, suggested that the enzyme was a unique, acid–thiol–arginyl protease. Here we have assayed for this pro-opiocortin converting activity in the intermediate lobe and the neural lobe separately. We show that purified secretory granules from both rat intermediate and neural lobes contain a pro-opiocortin converting activity that is specific for pairs of basic amino acids, similar to that reported for neurointermediate lobe granules [9]. However, the converting activity in the 2 lobes differed in their preferences for the paired basic amino acid cleavage sites in the pro-opiocortin molecule.

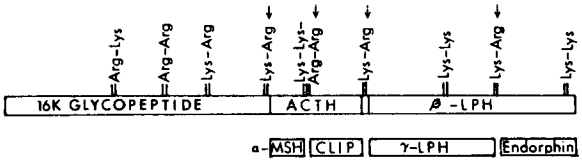

Fig.1. Structure of pro-opiocortin showing the arrangement of the different products in the molecule and the location of the basic amino acid pairs, as found in the bovine sequence [5].

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2. Materials and methods

2.1. *Animals*

Female rats (Osborne-Mendel strain) (200–250 g body wt) were obtained from the National Institutes of Health (Bethesda MD). Adult toads (*Xenopus laevis*), 40–70 g were purchased from NASCO Biological Supplies (Fort Atkinson WI) and maintained in a black plastic aquarium at 22°C with constant light for 15–21 days before use.

2.2. *Preparation of labeled toad pro-opiocortin (ACTH/endorphin precursor)*

Toad neurointermediate lobes were preincubated at 22°C in amphibian ringer [9] for 1 h and then 'pulse' incubated for 1.5 h in amphibian ringer containing 19.5 μM [^{3}H]phenylalanine (New England Nuclear Corp. (Boston MA), spec. act. 13.7 Ci/mmol). After the pulse, the lobes were homogenized in 0.1 M HCl and the proteins precipitated with 10% trichloroacetic acid. Labeled pro-opiocortin was purified by

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acid gel electrophoresis, omitting urea in the gel [9]. Following electrophoresis of the trichloroacetic acid-precipitated proteins, the gel was sliced and each gel slice was eluted with 0.02 M HCl/0.025 mg bovine serum albumin/ml. The eluant from the gel slice(s) containing the pro-opiocortin was lyophilized for use as substrate. The labeled pro-opiocortin substrate was 80–85% pure as determined by immunoprecipitation with adrenocorticotropin (ACTH) and endorphin antisera.

2.3. *Preparation of secretory granules from the intermediate and neural lobes*

Neurointermediate lobes were removed from 50 rats and each neural lobe was separated from the intermediate lobe by microdissection. The intermediate and the neural lobes were then homogenized separately in 2 ml 0.25 M sucrose/10 mM Tris–HCl, pH 7.4 at 0°C using a Potter-Elvehjem-type homogenizer. The homogenate was then subjected to differential centrifugation as in [9,10]. Five different fractions (P_I–P_{IV} and S) were generated by this procedure. Each fraction was assayed for α-MSH in the intermediate lobe [11] and arginine–vasopressin (AVP) in the neural lobe [10], β-glucuronidase (lysosomal marker) [10] and monoamine oxidase (MAO, mitochondrial marker) [12]. The P_{III} fractions from the intermediate and neural lobes were found to have the highest content of α-MSH (77%) and AVP (35%), respectively, suggesting that they are granule-enriched fractions. The P_{III} fraction from the 2 lobes was taken for further purification by discontinuous sucrose density gradient centrifugation as in [10]. After centrifugation, the centrifuge tube was pierced and fifteen 25 drop fractions were collected from the sucrose density gradient. Each fraction was analyzed for α-MSH or AVP, β-glucuronidase and MAO. The analyses indicated that fraction 6 contained highly purified secretory granules (see fig.2) and it was taken for analysis of pro-opiocortin converting activity.

2.4. *Assay of pro-opiocortin converting activity in rat intermediate and neural lobe secretory granules*

The secretory granules from fraction 6 of the sucrose gradient for each of the lobes were osmotically lysed and then adjusted to pH 5.0 with ammonium acetate buffer (final conc. 0.1 M). The labeled pro-opiocortin substrate was dissolved in 0.1 M ammonium acetate (pH 5) buffer. The secretory granule lysate containing 20–25 μg protein was then incubated together with labeled pro-opiocortin (~5000 cpm) in 200 μl total vol. for 5 h at 37°C. In assays done at pH 7.4, granules were lysed and incubations carried out using 10 mM Tris–HCl buffer. At the end of the incubation the reaction was stopped by the addition of HCl to 0.2 M HCl final conc. Aliquots of the incubation mixture were taken for analysis by acid–urea gel electrophoresis. For the identification of the processed products, aliquots of the incubation mixture were quantitatively immunoprecipitated with excess ACTH or β-endorphin (RB 100) antisera. The ACTH antiserum used is specific for the N-terminus of ACTH, and crossreacts with pro-opiocortin, $ACTH_{1-39}$, $ACTH_{1-24}$ and α-MSH but not $ACTH_{17-39}$. The β-endorphin antiserum used is specific for the C-terminus of β-LPH (beyond residue 77) and crossreacts with pro-opiocortin, β-LPH and β-endorphin but not α-endorphin [13]. The immunoprecipitates were analyzed by acid–urea gel electrophoresis as in [3,9], except that *Staphylococcus aureus* Cowan I cells (IgGsorb, The Enzyme Center Inc. (Boston MA)) was used to precipitate the antigen–antibody complexes. The N-terminal 16 000 M_r glycopeptide product was identified by M_r-value and ability to bind concavalin A [14]. The M_r of processed products were determined by SDS gel electrophoresis [15].

2.5. *Inhibitor studies*

Proteolytic inhibitors, *p*-chloromercuribenzoate (PCMB), diisopropylfluorophosphate (DFP), and chloroquine were purchased from Sigma Chemicals Co. (St Louis MO). Leupeptin and pepstatin A were gifts from Drs W. Troll and Umezawa (New York NY). The concentration of the inhibitors is reported in the text.

3. Results

3.1. *Analysis of the purity of the secretory granules from intermediate and neural lobes*

The results of isolation of the intermediate and neural lobe secretory granules used in this study is shown in fig.2. In the intermediate lobe (fig.2A), fraction 6 was determined to be the purest α-MSH containing granule fraction since it contained no mitochondrial MAO activity and had the least contamination with lysosomal β-glucuronidase activity (<0.5% of that present in the original intermediate lobe). In the neural lobe (fig.2B), fraction 6 was also

the purest of the 3 hormone (AVP) containing granule fractions. This fraction contained no mitochondrial MAO activity and had minimal contamination with lysosomal β-glucuronidase activity (<0.8% of that present in the original neural lobe tissue). In both the intermediate and neural lobes, the MAO and β-glucuronidase activities were maximally concentrated in fraction 11. Thus the granules in fraction 6 of the intermediate and neural lobe preparations were used to assay for the pro-opiocortin converting activity.

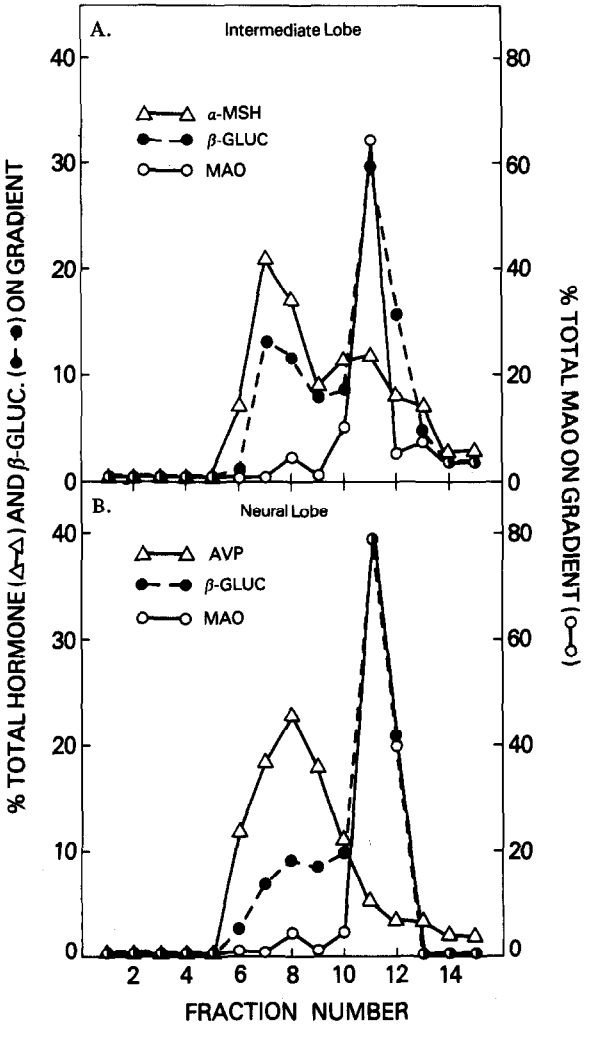

Fig.2. Distributions of hormone (α-MSH or AVP) and enzyme activities (MAO, monoamine oxidase; β-Gluc, β-glucuronidase) in the P_{III} (granule) fraction of intermediate (A) and neural lobe (B) after sucrose density-gradient centrifugation (see methods). The data are expressed on the ordinate as a % of the total hormone content (or enzyme activity) in the P_{III} fraction which was found in each gradient fraction after density gradient centrifugation (abscissa).

3.2. *Incubation of toad pro-opiocortin with intermediate lobe granule lysates*

[^{3}H]Phenylalanine labeled pro-opiocortin was incubated with and without lysed secretory granules (SG) at pH 5 or pH 7.4 for 5 h at 37°C. Incubations with SG at pH 5 resulted in 83.3% conversion of pro-opiocortin while essentially no conversion was observed at pH 7.4 (not shown). Incubations in the absence of SG at pH 5 showed no conversion of the prohormone (fig.3A), but in the presence of SG, several products were formed (fig.3). The toad pro-opiocortin was cleaved to form a 21 000 M_r (21 kM_r) ACTH, 2 forms of 13 000 M_r (13 kM_r) ACTH and α-MSH as identified by immunoprecipitation with ACTH antiserum (fig.3A,B (●)). A 16 000 M_r glycopeptide cleavage product (16 kM_r GP) was detected and identified as the N-terminal glycopeptide of pro-opiocortin by its size, binding to con A and lack of immunoprecipitation by ACTH or endorphin antisera (fig.3A). Cleavage products corresponding to β-LPH and 2 forms of β-endorphin were detected by immunoprecipitation with β-endorphin antiserum (fig.3A,B (○)). One of the β-endorphin-like peptides (β-END) migrated with a mobility close to that of the β-endorphin standard, while the other (β-ELP) migrated at slice 45. The β-endorphin immunoprecipitation data (fig.3B (○)) also revealed a large β-endorphin related product (21 kM_r). This molecule was found to have the ACTH sequence as well, as determined by sequential immunoprecipitation with ACTH and endorphin antisera, i.e., immunoprecipitation with endorphin antiserum after immunoprecipitation with ACTH antiserum resulted in the absence of the 21 kM_r endorphin-related peak. It is therefore referred to as the ACTH/β-LPH product. This product accounts for 35–45% of the radioactivity in the 21 kM_r ACTH immunoprecipitated peak (fig.3B (●)).

3.3. *Incubation of toad pro-opiocortin with rat neural lobe granule lysates*

Incubation of [^{3}H]phenylalanine labeled pro-opiocortin for 5 h at 37°C with lysed neurosecretory granules (NSG, fraction 6) at pH 5.0 resulted in 81.9% conversion of the substrate, but there was essentially no processing when the incubation was at pH 7.4 (not shown). Incubations at pH 5.0 for 5 h in the absence of NSG showed no conversion of pro-opiocortin but in the presence of NSG, several products were formed (fig.4). Two products were identified by immunoprecipitation, with ACTH antiserum (fig.4B (●)) as a large

form of ACTH having $M_r \sim 13\,000$ (13 kM_r) and α-MSH (slice 53). Another product was identified as the 16 000 M_r N-terminal glycopeptide (16 kM_r GP) of pro-opiocortin by its binding to con A, M_r-value and lack of immunoprecipitation by either ACTH or endorphin antisera (compare fig.4A,B). Three of the products formed were shown to be immunoprecipitated by β-endorphin antiserum (fig.4B (○)). One of them was identified as β-LPH on the basis of its size (fig.4A,B (○)). Another migrated at slice 45 (fig.4B (●)), and is referred to as a β-endorphin-like peptide (β-ELP). A 21 000 M_r (21 kM_r) anti-β-endorphin

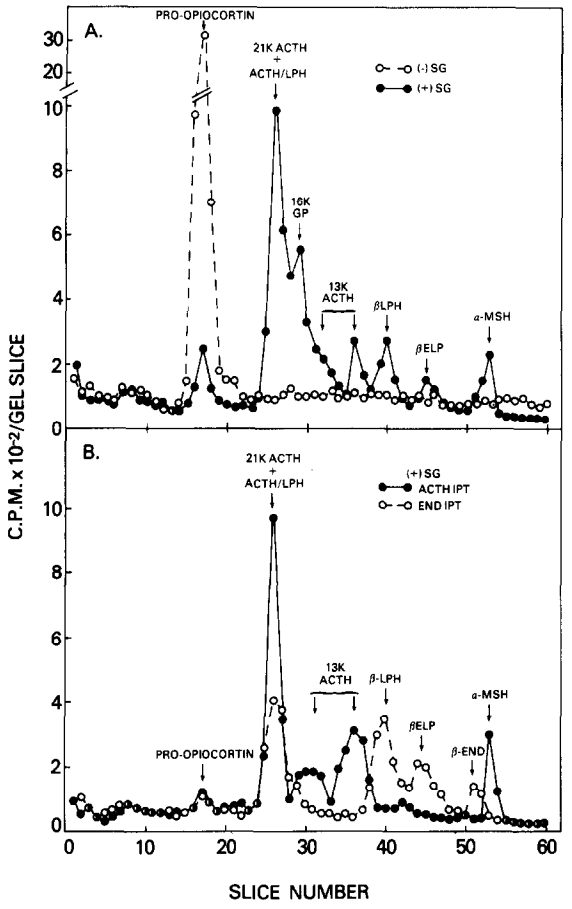

Fig.3. Conversion of [^{3}H]phenylalanine labeled pro-opiocortin, at pH 5.0, by lysed rat intermediate lobe secretory granules (SG). (A) Acid–urea gel profiles of labeled proteins and peptides following a 5 h incubation (pH 5.0, 37°C) of the [^{3}H]pro-opiocortin in the absence (○---○) and presence (●——●) of lysed secretory granules. Note that conversion of the pro-opiocortin to peptide products occurs only in the presence of secretory granule lysate. The identity of the products cleaved from pro-opiocortin is indicated above the peak. (B) Immunological identification of peptide products seen in (A). An equal aliquot of the incubate as used for (A) was immunoprecipitated with ACTH (●——●) or β-endorphin antisera (○---○) followed by acid–urea gel electrophoresis. IPT, immunoprecipitate. Each profile shown in fig.3A,B is representative of 3 and 2 expt, respectively. The percentage of total cpm in each identified peak varied ⩽15% between the duplicate (triplicate) experiments.

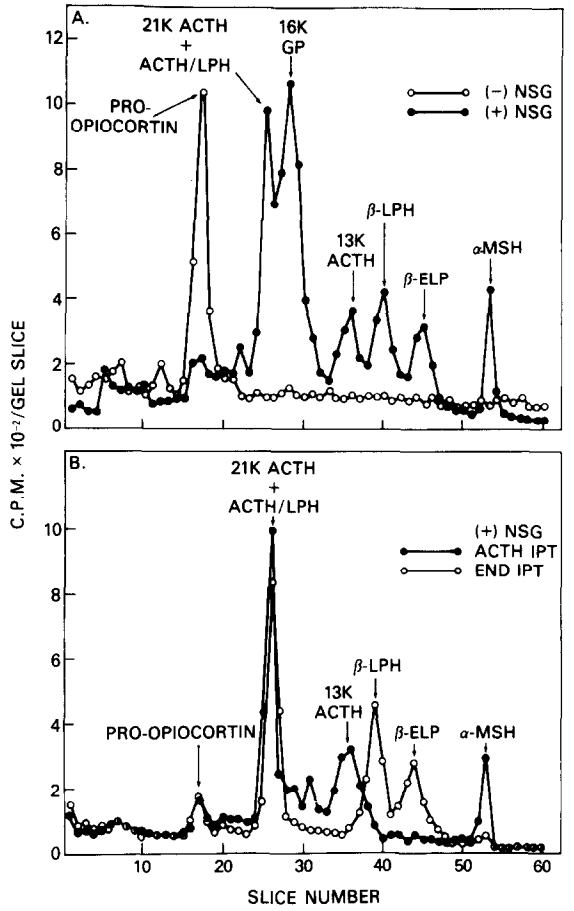

Fig.4. Conversion of [^{3}H]phenylalanine labeled pro-opiocortin, at pH 5.0, by lysed rat neural lobe secretory granules (NSG). (A) Acid–urea gel profiles of labeled proteins and peptides following a 5 h incubation (pH 5.0, 37°C) of the [^{3}H]pro-opiocortin substrate in the absence (○---○) and presence (●——●) of lysed secretory granules. Note that conversion of the pro-opiocortin to peptide products occurs only in the presence of secretory granule lysate. The identity of the peptide derived from pro-opiocortin is indicated above the peak. (B) Immunological identification of peptide products seen in (A). An equal aliquot of the incubate as used in (A) immunoprecipitated with ACTH (●——●) or β-endorphin antisera (○---○), followed by electrophoresis on acid–urea gels. IPT, immunoprecipitate. Each profile shown in fig.4A,B is representative of 3 and 2 expt, respectively. The percentage of total cpm in each identified peak varied ⩽15% between the duplicate (triplicate) experiments.

immunoprecipitated product was also detected (fig.4B (○)). This molecule is referred to as ACTH/β-LPH since it is immunoprecipitated by both ACTH and endorphin antisera. The presence of both the ACTH and endorphin antigenic determinants on this molecule was further confirmed by sequential immunoprecipitation. All of the radioactivity immunoprecipitated by the β-endorphin antiserum in the 21 kM_r peak appears to be due to the ACTH/β-LPH product, and accounts for 80% of the total ACTH immunoprecipitable activity in the peak.

3.4. *Characterization of the pro-opiocortin converting activity in intermediate and neural lobe secretory granules*

A comparison of the nature of the pro-opiocortin converting activity in the rat intermediate and neural lobe secretory granules was conducted using different protease inhibitors. Table 1 shows that PCMB, a thiol protease inhibitor and leupeptin, an arginyl protease inhibitor [16] were effective in inhibiting the converting activity in both lobes. Pepstatin A was also effective in inhibiting the converting activity in the intermediate and neural lobes. Chloroquine, an inhibitor of cathepsin B [17] and DFP, an inhibitor of serine proteases were without effect on the converting activity of either lobe.

4. Discussion

Here, we have demonstrated the presence of a pro-opiocortin converting activity in the secretory granules of both intermediate and neural lobes, that is similar to that reported for the total neurointermediate lobe [9]. The products formed in both lobes, suggests that cleavages occurred at the paired basic amino acid residues (see fig.1, [9]). These products did not undergo further degradation in the presence of granule lysate for ⩾18 h (unpublished). The two lobes, however, showed some difference in the products formed. The intermediate lobe granule lysate appears to preferentially cleave pro-opiocortin at the paired basic amino acid residues between ACTH and β-LPH (see fig.1) yielding predominantly 21 kM_r ACTH (16 kM_r glycopeptide + ACTH) and β-LPH (fig.3). In contrast, the neural lobe granule lysate appears to cleave primarily at the paired basic amino acid residues between the 16 kM_r glycopeptide and ACTH (see fig.1), yielding ACTH/β-LPH and the N-terminal 16 kM_r glycopeptide as major products (fig.4). Note the larger 16 kM_r glycopeptide and 21 kM_r anti-β-endorphin immunoprecipitated peaks in the neural lobe incubate as compared to that of the intermediate lobe (cf. fig.3,4). The second difference is that fewer processed products were formed by the granule lysates of the neural lobe versus intermediate lobe (fig.3,4). While pro-opiocortin incubated with intermediate lobe granule lysate was processed to 2 forms of 13 kM_r ACTH, 2 forms of β-endorphin (including one that co-migrated close to synthetic β-endorphin), β-LPH and α-MSH; neural lobe granule lysates cleaved the substrate to primarily one form of 13 kM_r ACTH and β-endorphin (β-ELP), β-LPH and α-MSH. Formation of the β-endorphin product that co-migrated with synthetic β_h-endorphin occurred only with the intermediate lobe granule lysate (fig.3) but not with the neural lobe (fig.4). All of the cleavage products formed by the rat intermediate lobe granule lysates are similar in electrophoretic mobility, on acid–urea gels, to those synthesized by the toad intermediate lobe [3,18]. However, the 21 kM_r ACTH/β-LPH molecule, the major product cleaved by the rat neural lobe granule lysate is not a significant product in the toad intermediate lobe [3,18].

While there are subtle differences in the specificity of the cleavage of pro-opiocortin by the granules from the intermediate and neural lobes, a comparison of the pH optima and inhibitor profiles of the activities suggest that the 2 enzymes are very similar. The 2 activities are greater at pH 5.0 than pH 7.4, suggesting that they are acid proteases. The inhibitor studies (table 1) show that the converting activities from both lobes are inhibited by the same agents, i.e., PCMB (a thiol protease inhibitor), leupeptin (an arginyl protease inhibitor [16]) and pepstatin A (an inhibitor that acts by formation of hydrophobic and hydrogen bonds with the enzyme [19,20]). DFP, a

Table 1
Effect of proteolytic inhibitors on pro-opiocortin conversion by intermediate and neural lobe secretory granules

Inhibitor	% Inhibition[a]	
	Intermediate lobe	Neural lobe
DFP (1 mM)	8.0 ± 3.8 (2)	12.0 ± 5.2 (2)
PCMB (1 mM)	95.5 ± 4.5 (3)	104.0 ± 7.0 (3)
Leupeptin (2 mM)	60.5 ± 3.5 (2)	63.5 ± 3.0 (2)
Chloroquine (100 μM)	4.0 ± 2.0 (2)	9.0 ± 1.7 (2)
Pepstatin A (1 mM)	93.0 (1)	88.2 (1)

[a] Mean ± standard deviation; no. expt is shown in parenthesis

serine protease inhibitor and chloroquine, a cathepsin B inhibitor [17] were without effect on the converting activities in either lobe. This inhibitor profile suggests that the pro-opiocortin converting activities in the intermediate and neural lobe granules are both due to an acid–thiol–arginyl protesase, distinct from cathepsin B, as suggested from our studies on the rat neurointermediate lobe [9]. The inhibition by pepstatin A suggests that the converting enzymes may have secondary hydrophobic and/or hydrogen-bonding sites that are involved in enzyme–substrate interaction [19,20]. Inhibition of converting activity by leupeptin, a hydrophobic molecule, occurs only at concentrations higher than that necessary to block trypsin activity [19]. This raises the possibility that the inhibition by leupeptin may not be due to specific binding at the active site as with trypsin, but rather may be due to a secondary effect, e.g., by binding to hydrophobic sites on the enzyme. The characteristics of the pro-opiocortin converting activity in the intermediate and posterior pituitary are also similar to the converting activities reported for pro-insulin, pro-glucagon and pro-somatostatin in the pancreatic islet granules of anglerfish [21,22]. Thus, there may exist a family of prohormone converting activities that are very similar in nature but differ slightly in their specificities.

In conclusion, we have shown that the intermediate lobe and neural lobe granules both contain an acid–thiol–arginyl protease that cleaves at paired basic amino acid residues, yet show subtle differential preferences for the paired basic amino acid cleavage sites of pro-opiocortin. This difference is perhaps not surprising since pro-opiocortin is not a natural substrate in the neural lobe. The converting activity in the neural lobe granules is probably tailored to cleave the prohormones for vasopressin and oxytocin synthesized in the hypothalamo–neurohypophysial system [23–25]. It is possible that such subtle differences in the specificity of the cleavage process of the converting activities in the 2 lobes may be dictated by the conformation of the enzymes as well as the substrate.

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